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The Role of Nucleotide Excision Repair in Restoring Replication Following UV-Induced Damage in Escherichia coli

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The Role of Nucleotide Excision Repair in Restoring Replication Following UV-Induced
Damage in *Escherichia coli*

by

Kelley Nicole Newton

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requirements for the degree of

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in
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Thesis Committee:
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ABSTRACT

Following low levels of UV exposure, *Escherichia coli* cells deficient in nucleotide excision repair recover and synthesize DNA at near wild type levels, an observation that formed the basis of the post replication recombination repair model. In this study, we characterized the DNA synthesis that occurs following UV-irradiation in the absence of nucleotide excision repair and show that although this synthesis resumes at near wild type levels, it is coincident with a high degree of cell death. We confirm that the replication occurring under these conditions involves extensive levels of strand exchange. However, cells undergoing this form of replication accumulate strand exchange intermediates that fail to resolve into discrete molecules, resulting in grossly filamentous, multinucleate cells. Taken together the results demonstrate that the DNA synthesis that occurs in UV-irradiated nucleotide excision repair mutants is aberrant and suggests that post replication repair is not an efficient mechanism to promote survival in the absence of nucleotide excision repair.

The role that nucleotide excision repair plays in the recovery of replication following UV-induced DNA damage was further characterized by examining the specific role of UvrD in processing and restoring UV-arrested replication forks. UvrD is a helicase with functions associated with nucleotide excision repair and replication. UvrD catalyzes the removal of the damaged region by nucleotide excision repair proteins and removes the stretch of DNA incised during methyl-directed mismatch repair during replication. Recent biochemical studies have led to the proposal that UvrD may promote fork regression and facilitate resetting of the replication fork following arrest. However, the molecular activity of UvrD at replication forks *in vivo* has not been directly

examined. In this study, we show that UvrD is required for DNA synthesis to recover. However, in the absence of UvrD, the displacement and partial degradation of the nascent DNA at the arrested fork occurs normally. In addition, damage-induced replication intermediates persist and accumulate in *uvrD* mutants in a manner that is similar to that observed in other nucleotide excision repair mutants. These data indicate that following arrest by DNA damage, UvrD is not required to catalyze fork regression *in vivo* and suggest that the failure of *uvrD* mutants to restore DNA synthesis following UV-induced arrest relates to its role in nucleotide excision repair.

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Mom, it's all been said. This one's for you...

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CHAPTER I

INTRODUCTION

Cells continuously encounter sources of DNA damage, both from the environment and as a consequence of their own metabolism. This damage can be a beneficial source of the genetic variability necessary to drive evolution. However, the inability to properly deal with DNA damage typically has deleterious outcomes for the cell. The threat of DNA damage is especially significant when encountered by actively replicating cells. Possible deleterious outcomes include the induction of mutations through the misincorporation of nucleotides when copying damaged templates, rearrangements, duplications, or deletions when replication is disrupted and resumes from the wrong site and lethality if the cell is unable to overcome the block to replication. UV light (254 nm) generates two lesions in DNA that block replication, cyclobutane pyrimidine dimers and 6,4-photoproducts, making it a useful model for the study of DNA damage [1, 2]. A number of disease states result from the mutation of genes required to process UV damage, including xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy, underscoring the biological significance of these repair mechanisms. The symptoms of these diseases range significantly. Xeroderma pigmentosum (XP) is caused by mutations in any one of seven genes associated with nucleotide excision repair. XP cells are unable to repair UV-induced lesions and patients with this disease are extremely sensitive to UV light and highly susceptible to skin cancers [3, 4]. A variant of XP, XP-V, also exists in which the same phenotypic characteristics result from a defect in translesion synthesis [5]. Cockayne syndrome (CS) is characterized by neurological defects, short stature, premature aging and photosensitivity. CS results from mutations in

the transcription coupled repair genes *CSA* and *CSB*, and cells from CS patients are unable to recover RNA synthesis following UV exposure [6, 7]. The clinical features of trichothiodystrophy (TTD) are diverse. The unifying characteristic is sulfur deficient brittle hair and other symptoms may include developmental and neurological defects and photosensitivity. TTD is associated with mutations affecting transcription factor TFIIH, which is associated with both nucleotide excision repair and transcription [8, 4]. While these three disease states have complex and diverse phenotypes, all are associated with defects in genes belonging to the nucleotide excision repair (NER) pathway[4].

Escherichia coli has homologs for both nucleotide excision repair and transcription coupled repair proteins. These processes are also highly conserved between organisms[7], making it a suitable model for characterizing the mechanism by which these pathways process DNA damage in humans. In this study, we used *E. coli* to characterize two aspects of the nucleotide excision repair process in replicating cells. The first examines how the presence or absence of nucleotide excision repair affects the recovery and completion of ongoing replication following UV damage. The second specifically examines how the UvrD helicase, which has roles in replication, mismatch repair, and the final steps of nucleotide excision repair, affects the recovery of replication following UV-induced DNA damage.

When replication encounters DNA damage before repair can occur, the cell is presented with a critical challenge. Replication of the damaged site is likely to render the region single stranded, resulting in the inhibition of nucleotide and base excision repair enzymes [9]. Furthermore, failure to accurately duplicate the region will result in mutagenesis, whereas failure to overcome the block to replication will result in lethality.

Following exposure to UV-light, wild type cells undergo a transient arrest of DNA synthesis during which the stalled replication fork must be processed and maintained prior to the resumption of replication[10]. In *E. coli*, this process is dependent on the RecFOR pathway (Figure 1.1) [10, 11]. The nascent DNA of the lagging strand at the arrested fork is degraded through the combined action of RecQ, a 3'-5' helicase, and RecJ, 5'-3' exonuclease[12, 13]. This degradation is limited in the presence of the RecFOR proteins[11], which load RecA onto single stranded regions of DNA at the arrested fork. Both *in vitro* and *in vivo* evidence suggest that RecA catalyzes strand exchange, effectively moving the branch point of the fork backward and restoring the region containing the offending lesion to a double stranded form[14, 15]. Current evidence suggests that the primary pathway of recovery involves the nucleotide excision repair machinery, which is then able to access the offending lesion and effect repair [10, 16].

In the absence of nucleotide excision repair, the resumption of DNA synthesis is severely impaired and delayed [10, 17, 18]. The eventual recovery of replication is associated with high levels of mutagenesis, strand exchange and cell lethality[10, 17, 18]. In prokaryotes, NER is carried out by UvrA, -B, -C and -D and cells deficient in any one of these genes are extremely UV-sensitive[19-21]. DNA helix distorting lesions are recognized by a heterotetramer consisting of UvrA₂UvrB. Once a lesion is bound, UvrA disassociates from the complex and UvrB binds to the damaged site. UvrB possesses weak helicase activity that is thought to promote local unwinding of the DNA strands, allowing for the recognition and binding by two molecules of UvrC[22, 23]. The UvrC subunits catalyze both the 3' and 5' incisions surrounding the lesion, leaving a 12-14 bp

oligonucleotide. A homologue of UvrC, Cho, is also capable of substituting for one UvrC subunit and creating the 3' incision[24]. Cho and UvrC bind to different domains of UvrB and Cho makes a 3' incision that is 4 nt further away from the damaged site than that made by UvrC. Consequently, Cho may act coordinately with UvrC to catalyze the incision of certain lesions, increasing the substrate range of NER. Following incision, the UvrD helicase both excises the damaged strand and releases the UvrBC complex from the DNA, creating a gap that is filled in by DNA polymerase I and sealed by DNA ligase[9, 25].

Nucleotide excision repair in eukaryotic cells is mechanistically similar to the process in prokaryotes. The damaged site is recognized by the XPC-HR23B complex[26, 27], which in turn recruits the proteins TFIIH, XPA, and RPA [28]. TFIIH contains two subunits, XPB and XPD, which catalyze DNA unwinding in the 3' to 5' direction and 5' to 3' direction, respectively. The dual helicase activity of TFIIH results in the formation of an "open complex" with single stranded character surrounding the damage site[29]. The exact function of XPA is unknown but it is thought to stabilize the open complex and is required for NER in eukaryotes[4]. RPA is a single-stranded binding protein that both stabilizes the ssDNA in the open complex and protects it from nucleases[30]. After the formation of the open complex the endonuclease XPG and the XPF-ERCC1 complex are recruited and catalyze dual incisions around the damage site, generating a 30 nt long gap[31, 32]. This gap is filled in by either polymerase δ or polymerase ϵ and the DNA ligated by DNA ligase 1 [33].

A subpathway of NER, termed transcription coupled repair (TCR) was discovered upon the observation that UV-induced DNA damage is more rapidly removed from

actively transcribed regions of the genome[34]. Unlike global genome repair (GGR), in which DNA damage is recognized and removed from the entire genome, TCR is triggered by the stalling of RNA polymerase at the damaged site. The stalled RNAP is then bound by the transcription repair-coupling factor, Mfd. Mfd contains both an ATP dependent helicase domain that allows it to translocate and release the RNAP and a region of homology with UvrB that enables it to recruit UvrA [7]. Following the recruitment of UvrA to the damaged region, the lesion is excised and the resulting gap filled as previously described for GGR[35, 7]. Similarly, TCR in eukaryotes is thought to occur when the transcription-repair coupling factor CSB, coordinately with CSA and XPG, recognizes the stalled RNA polymerase [36, 37]. TFIIH, XPA, and RPA are then recruited and the lesion is repaired in the same manner as in GGR.

Nucleotide excision repair results in the removal of the offending lesion and is considered to be an error free process. However, there are damage tolerance mechanisms in which lesions persist such as translesion DNA synthesis and post replication recombination repair. During translesion synthesis, damage inducible DNA polymerases incorporate nucleotides opposite the damaged site, thus bypassing the lesion. In *Escherichia coli* there are three translesion polymerases: Pol II, Pol IV, and Pol V. These polymerases are encoded by *polB*, *dinB*, and *umuCD* respectively [38, 39, 40]. The ability to bypass lesions using these polymerases is coincident with an increased mutation rate [41]. Pol II possesses a 3'-5' exonuclease [42] and is a relatively high fidelity translesion polymerase when compared to Pol IV and Pol V, which are Y-class polymerases and therefore lack exonuclease and proofreading ability [43, 44, 45]. However, only Pol V significantly contributes to survival, the recovery of replication, and

mutagenesis following UV-induced damage [46, 47]. Under normal conditions, Pol V does not significantly contribute to the rate at which replication recovers following UV-exposure. However, in the absence of nucleotide excision repair or RecJ mediated replication fork processing, PolV becomes essential for replication to resume[16]. It is likely, therefore, that translesion synthesis does not represent the predominant pathway for dealing with UV-induced damage.

Many of the protein products involved in nucleotide excision repair and translesion synthesis are upregulated following DNA damage during a process known as the SOS response[48]. Genes that are under transcriptional control of the SOS response contain a 20 bp consensus sequence to which the repressor, LexA, binds [49, 50]. In the presence of DNA damage, single stranded regions of DNA are generated following the disruption of replication forks. RecA binds to these regions of single stranded DNA and becomes conformationally active, acting as a coprotease that promotes the autocatalysis of LexA. This results in the upregulation of over 40 genes associated with DNA repair, translesion synthesis and cell cycle regulation [51, 52]. The protolytically active form of RecA also participates in the cleavage of UmuD to UmuD', the form active in translesion synthesis [53].

The role RecA plays in the generation of the SOS response makes it a crucial component of the DNA damage response. However, RecA is an important component of several other cellular processes. As described previously, RecA binds to the single stranded DNA at stalled replication forks and serves to protect, maintain, and likely catalyze the forks regression [14, 54, 55]. RecA pairs the single stranded DNA with the homologous duplex DNA of the sister chromatid at the stalled fork. This creates a three

stranded DNA filament, maintained by RecA, that is resistant to exonucleolytic attack[12, 11]. When the replication machinery encounters a blocking lesion in *recA* mutants, replication does not recover and a rapid degradation of the genomic DNA takes place[11, 54]. These results are consistent with a model in which RecA protects and maintains the stalled replication fork until the lesion can be removed or bypassed and replication can resume.

The strand-pairing activity of RecA is also required to bring together homologous DNA molecules during sexual events [56]. RecA was originally identified during a screen for conjugation deficient mutants. Cells lacking RecA are able to transfer DNA during conjugation but are unable to form recombinant molecules[57]. A subsequent study determined that *recA* mutants are hypersensitive to UV and X-ray irradiation [58]. To explain the hypersensitive phenotype, a model was proposed in which recombination is also required during asexual cell cycles to carry out repair[58]. The model, termed post-replication repair, involves a process by which lesions are skipped over by the DNA polymerase, generating single stranded gaps in the nascent DNA. These gaps are subsequently paired with the undamaged homologous duplex DNA by RecA and the genome is reconstructed through the creation of recombinant molecules [17, 18]. Consistent with this model, the amount of DNA synthesis following low doses of UV light in *uvrA* mutants is similar to that in wild type cells[58, 59]. Furthermore, following UV irradiation in *uvrA* mutants the newly synthesized DNA is fragmented and high levels of strand exchange are observed[17, 18],

However, other observations do not necessarily support the view that recombination is an effective mechanism that promotes survival following UV-induced

damage. Virtually all studies characterizing post-replication recombination repair have been done in nucleotide excision repair mutants[17, 18, 60]. However, survival is synergistically enhanced when both recombination and repair genes are functional, suggesting that these proteins normally act in a similar pathway. Further, high rates of strand exchange are only detected in the absence of nucleotide excision repair, conditions where high levels of lethality are also observed[17, 18]. In its presence, exchanges are difficult to detect and lethality is significantly diminished. Finally, a number of observations have shown that replication does not efficiently recover in the absence of genes required for nucleotide excision repair [10, 16] suggesting that the replication machinery does not efficiently skip over lesions during replication as proposed.

In this thesis, I examine two specific aspects of nucleotide excision repair function in replicating cells exposed to UV-irradiation. In Chapter II, I characterize the nature of the DNA synthesis that occurs after low doses in *uvrA* mutants to address whether RecA, in the absence of nucleotide excision repair, is able to promote cell survival in the face of damage.

In Chapter III, I specifically characterize the role of UvrD in processing and restoring replication forks arrested following UV-damage. UvrD is a helicase with functions associated with nucleotide excision repair and replication. UvrD catalyzes the removal of the damaged region following excision by nucleotide excision repair proteins and removes the stretch of DNA incised during methyl-directed mismatch repair during replication. [61-63] A number of studies have shown this gene can affect both replication and recombination efficiency [64][65-68]. Recent biochemical studies have proposed that UvrD catalyzes fork regression following the disruption of the replication

machinery[69][70-72]. However, the cellular role of UvrD at arrested replication forks, *in vivo*, has not been previously examined. This characterization is performed in Chapter III.

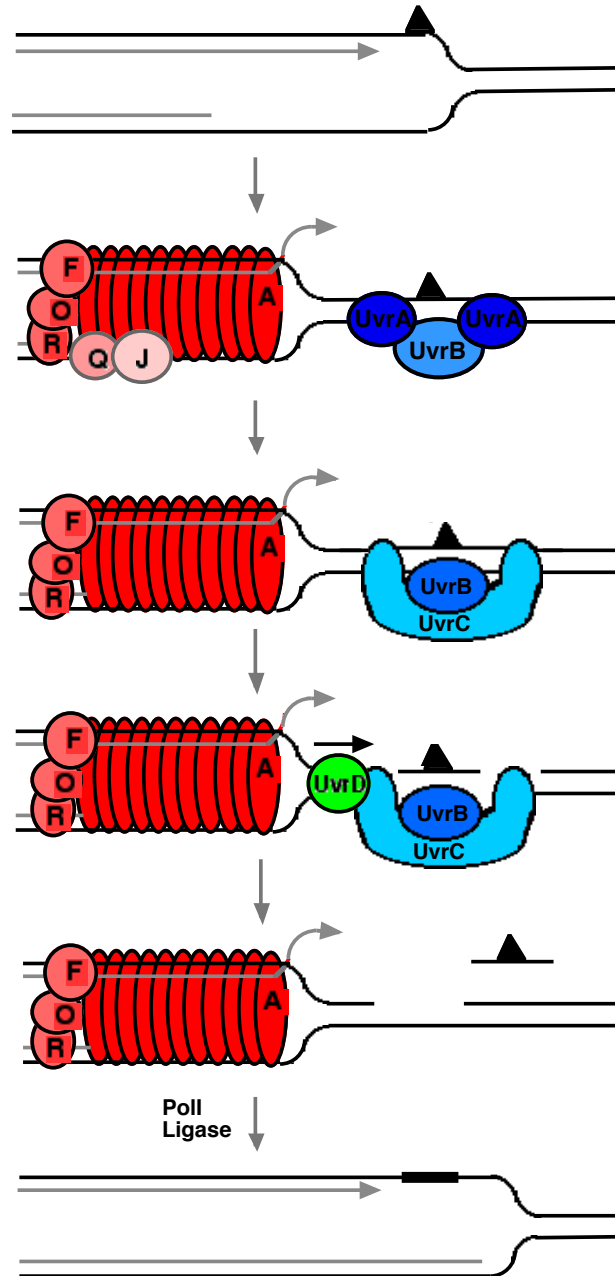


Figure 1.1 Model for the recovery of replication in the presence of UV-induced DNA damage.

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CHAPTER II

THE POST-IRRADIATION DNA SYNTHESIS THAT OCCURS IN *uvrA* MUTANTS IS ABBERANT AND LEADS TO HIGH LEVELS OF INVIABILITY

ABSTRACT

Following low levels of UV exposure, *Escherichia coli* cells deficient in nucleotide excision repair (*uvrA6* mutants) recover and synthesize DNA at near wild type levels, an observation that formed the basis of the post-replication recombination repair model. In this study, we characterized the DNA synthesis that occurs following UV-irradiation in the absence of nucleotide excision repair and show that although synthesis resumes at near wild type levels, less than 1% of the cells survive to form viable cells. Using two-dimensional agarose gel analysis, we confirm that the replication occurring under these conditions involves extensive levels of strand exchange. However, cells undergoing this form of replication accumulate strand exchange intermediates that fail to resolve into discrete molecules, resulting in grossly filamentous, multinucleate cells. Taken together the results demonstrate that the DNA synthesis that occurs in UV-irradiated *uvrA6* mutants is aberrant and suggests that post-replication repair is not an efficient mechanism to promote survival in the absence of nucleotide excision repair.

INTRODUCTION

Accurate duplication of the genome is crucial to survival of any organism. Endogenous and exogenous sources of DNA damage can disrupt this process and result in mutagenesis, genomic instability, and lethality. Exposure to UV-irradiation (254 nm)

generates cyclobutane-pyrimidine dimers (CPDs) and pyrimidine-6-4-pyrimidone photoproducts (6-4PPs) that block the replication machinery [1] [2]. In *Escherichia coli*, the RecA protein is essential for survival in the face of such damage [3].

recA was originally identified in a screen for mutants deficient in the sexual process of conjugation. In the absence of RecA, bacteria were able to transfer DNA during conjugation but recipients were unable to undergo recombination [3]. Subsequent studies soon found that *recA* mutants were also hypersensitive to UV and X-ray irradiation[4]. Based on its known role in sexual recombination, a model was proposed in which RecA promotes recombination as a mechanism for repairing DNA damage [4], which was initially termed post-replication recombination repair[5] [6]. This model speculated that if DNA polymerases were able to skip over lesions during the course of replication, then the unreplicated lesions could be filled in via RecA-mediated strand exchanges with the undamaged homologous regions from sister chromatids. Support for this model came from three primary observations by subsequent studies, the interpretation of which is now appropriately being re-examined.

The first observation was based on the survival of *recA*, *uvrA*, and *recAuvrA* mutants following exposure to UV-irradiation. Whereas *recA* mutants could survive UV doses that produce approximately 20 lesions per cell and *uvrA* mutants could survive doses producing approximately 60 lesion per cell, *recAuvrA* double mutants were only able to survive doses producing approximately 2 lesions per cell[4, 7, 8]. This observation was used to suggest that *recA* and *uvrA* were epistatic, and that potential role of RecA in a recombinational repair process operated in a separate pathway from nucleotide excision repair and UvrA.

A second observation involved the inhibition of replication. Although it is established that UV-lesions block DNA synthesis in the absence of repair[1], IF low doses of irradiation were used, DNA synthesis could recover in the absence of *uvrA*. Furthermore, when the DNA was labeled at the time of UV-irradiation, a small amount of DNA made in the *uvrA* cells contained gaps[6]. These observations were used to support the view that the replisomes may be skipping over DNA lesions during replication. The third observation was that if low doses of UV-irradiation were used, elevated levels of strand exchanges were observed in the *uvrA* mutants, an observation that was used to suggest that recombination was occurring in these populations[5].

Taken together these three observations were used to support the post replication repair model. However, these observations are based on studies that took place in the absence of nucleotide excision repair and if re-examined in the context of what is occurring in a wild type cell following UV-irradiation, can be interpreted quite differently. Firstly, while *uvrArecA* double mutants have a decreased ability to survive following UV-induced damage when compared to either a *recA* or *uvrA* single mutant, genetic epistasis would predict that the effects of UvrA and RecA should be additive and they are not. If both RecA and UvrA are functional, cells are capable of surviving more than 3500 lesions per genome[4, 7, 8]. Thus, survival is synergistically and dramatically enhanced only when both genes are functional, indicating that the primary mechanism promoting survival in the cells occurs through a pathway that involves both RecA and UvrA function. The mis-interpretation that RecA acts independently of repair has had serious consequences on our understanding of RecA function, as virtually all subsequent

studies characterizing post-replication recombination repair have been performed in a nucleotide excision repair deficient background.

Secondly, while *uvrA* mutants continue to synthesize DNA following low doses of UV-irradiation, doses of UV-irradiation from which wild type cells are able to recover and fully resume replication completely inhibit DNA synthesis in cells lacking nucleotide excision repair [9]. Therefore, post replication recombination repair is unable to promote the recovery of replication following moderate doses of UV light in the absence of nucleotide excision repair. The recovery of replication from modest UV doses requires both UvrA and RecA function. Finally, few strand exchanges are observed following UV-irradiation in wild type cells[10], indicating that recombination does not contribute significantly to the repair of UV induced damage in a wild type context.

When the initial three observations are viewed in this light, it seems more likely that RecA and UvrA are acting together to repair UV-induced DNA damage and that the role of RecA in this process is not recombinational. Significant evidence suggests that RecA maintains and protects replication forks that have been stalled by lesions so that those lesions may be removed by nucleotide excision repair. In the absence of RecA, the genome is degraded following UV exposure and this degradation initiates from replication forks [10, 11]. Furthermore, two dimensional gel electrophoresis studies have shown that the intermediates indicative of fork regression and processing following UV-irradiation in wild type cells are absent in *recA* mutants[12]. *In vitro*, it has been shown that RecA catalyzes the pairing of single stranded DNA to homologous duplex DNA, a function that facilitates strand exchange [13-15]. Arrested replication forks also provide a substrate upon which RecA may act. However, it seems more likely that the primary

role of RecA's strand pairing activity in the recovery from UV-induced damage does not involve recombination but instead the stabilization and maintenance of stalled replication forks until the damage can be removed by nucleotide excision repair.

A remaining question from the original studies involving *uvrA* mutants is their continued DNA synthesis following low doses of UV-irradiation [6] [5]. As this synthesis is associated with high levels of strand exchange, it was originally interpreted as a product of post-replication recombination repair. Further, it seems to contradict the models in which the recovery of replication is coupled to the repair of the lesion. Here we characterize the nature of the synthesis occurring in these cells in order to better understand the function of the RecA protein in DNA repair. We confirm that the synthesis that occurs in the absence of repair is highly recombinogenic, but show that it is not productive, and leads to unbalanced, aberrant replication on the chromosome that leads to extensive filamentation and death in the cells in which it occurs.

MATERIALS AND METHODS

Bacterial Strains

All bacterial strains are in an SR108 background. SR108 is a *thyA36 deoC2* derivative of W3110[16]. HL921 (SR108 *sLR-recA306::Tn10*) and HL952 (SR108 *uvrA::Tn10*) have been described previously [17][10] [9]. CL23 (SR108 *uvrA6::Tn5*) was constructed by P1 transduction of Tn5 from HL759 into SR108.

DNA Accumulation and UV Cell Survivals

A Sylvania 15-watt germicidal lamp (254 nm) delivering an incident dose of 0.2 J J/m²/s was used for all irradiations. Fresh overnight cultures were diluted 1:100 in DGCthy medium (Davis medium supplemented with 0.4% glucose, 0.2% casamino acids and 10 µg/ml thymine) supplemented with 0.1 µCi/ml [³H]-thymine and grown to an OD₆₀₀ of 0.4 at 37°C in a shaking water bath. Half of each culture was irradiated with an incident dose of 6 J/m² and the other half was mock irradiated. At the times indicated, duplicate 0.2 ml samples were taken from each culture. These samples were added to 5% trichloroacetic acid to lyse the cells and precipitate the DNA. The DNA was filtered onto 2.4 cm Fisherbrand glass fiber filters and the amount of radioactivity on each filter was measured using a liquid scintillation counter. Immediately following irradiation and after four hours of recovery, 0.1 ml of each culture was taken to measure cell survival. Serial dilutions of each culture were plated in triplicate on Luria-Bertania plates supplemented with 10 µg/ml thymine and UV-irradiated with the indicated doses. Plates were grown overnight at 37°C and colonies were counted the following day.

Two-dimensional Agarose Gel Electrophoresis

Cells carrying the pBR322 plasmid were grown overnight in DGCthy medium containing 100 µg/ml ampicillin. One millimeter of this culture was pelleted, resuspended at a 1:100 ratio in 20 ml of DGCthy medium and grown in the absence of ampicillin at 37°C in a shaking water bath to an OD₆₀₀ of 0.5. The cultures were then UV-irradiated with 50 J/m² and, at the times indicated, 0.75 ml aliquots were transferred to an equal volume ice cold 2X NET (20 mM Tris pH 8, 20 mM EDTA pH 8, 200 mM

NaCl). These samples were then pelleted, resuspended in 0.14 ml of lysis buffer (1.5 mg/ml lysozyme, 0.5 mg/ml RNase A in 10 mM Tris, 1 mM EDTA) and incubated at 37°C for 30 minutes. Next, 10 µl of 20% Sarkosyl and 10 µl of 10 mg/ml proteinase K was added and the incubation was continued for 30 more minutes. The samples were then extracted twice with 4 volumes of phenol/chloroform/isoamyl alcohol (25:24:1), extracted once with 4 volumes of chloroform/isoamyl alcohol (24:1) and dialyzed against 200 ml of TE buffer (1mM EDTA, 2 mM Tris pH 8) for one hour on floating 37 mm Whatman 0.05 µm pore discs. The samples were digested with PvuII overnight, extracted with 2 volumes of chloroform/isoamyl alcohol (24:1) and loaded onto a gel. The gel was run at 25V for 16 hours in 1X TBE buffer (Tris-borate-EDTA pH 8). For the second dimension, the lanes were excised, rotated 90 degrees, recast in a 1% agarose gel with 1X TBE and the gel was electrophoresed at 200V for 7 hours. The DNA from the gels was transferred to N+ Hybond membrane using a Southern blot. The plasmid DNA was detected with an [α]dCT³²P (MP bio)-labeled pBR322 probe that was prepared using a nick translation protocol (Roche). Radioactivity was visualized and quantified using a Storm 840 Phosphoimager and ImageQuant software (Molecular Dynamics).

Fluorescence Microscopy

Fresh overnight cultures were diluted at a 1:100 ratio in DGCthy medium and incubated at 37° C until an OD₆₀₀ of 0.4 was reached. At this time half of each culture was UV-irradiated at a dose of 6 J/m² and the other half mock irradiated. Immediately following irradiation and after two and four hours of recovery at 37° C, 1 ml of each culture was pelleted and resuspended in 0.2 ml of 1X Davis medium containing 5 µg/ml 4', 6-

diamidino-2-phenylindole (DAPI). Following 5 minutes of staining, the cells were washed by pelleting and resuspension in 1X Davis medium twice. The cells were observed both using brightfield and fluorescence microscopy with an Axio Imager.M2 microscope (Zeiss).

RESULTS AND DISCUSSION

DNA synthesis in the absence of nucleotide excision repair does not promote cell survival.

In the absence of nucleotide excision repair, replication is severely inhibited and fails to recover from UV irradiation [1][9]. However, following low doses of UV-irradiation (2-5 J/m²), *uvrA* mutants retain an appreciable ability to restore synthesis to levels similar to that seen in unirradiated cells [18] [7]. This is in contrast to mutants deficient in the recombination protein, RecA, which do not continue to synthesize DNA following low doses of UV irradiation. To begin to characterize the nature of the DNA synthesis occurring under these conditions, we examined cell survival in wild type, *uvrA* and *recA* cultures following low dose UV exposure while simultaneously measuring the amount of replication that occurred. To monitor DNA synthesis, cultures grown in [³H]-thymine containing media were irradiated with 6J/m² and the total amount of [³H]-thymine in DNA was followed over time. The fraction of cells surviving these treatments was determined by comparing the colony forming units in irradiated and unirradiated cultures at 0 and 4 hours after irradiation. Following UV irradiation of wild type cells with 6 J/m², DNA synthesis continued to accumulate at a rate similar to that of the unirradiated culture and the survival remained close to 100% (Figure 2.1). Conversely,

following UV irradiation of *recA* cultures, no further DNA accumulation was observed and more than 99.9% of cells in the culture were rendered inviable. When we examined UV-irradiated cultures of *uvrA*, we observed that DNA synthesis recovered and DNA continued to accumulate, with 75.4% of the amount seen in unirradiated cultures four hours post irradiation. However, despite the significant amount of synthesis, more than 99% of the cells in the population were inviable. By four hours after irradiation, less than 3% of the culture was made up of viable cells, indicating that outgrowth of the few remaining survivors in the irradiated *uvrA* culture cannot account for the observed synthesis. The results demonstrate that the synthesis occurring in the absence of nucleotide excision repair is nonproductive and that the cells undergoing this form of synthesis are unable to survive.

Strand exchange intermediates accumulate and persist in the replicating DNA of UV-irradiated *uvrA* mutants.

Previous work has shown that the post irradiation DNA synthesis occurring in NER mutants is made up of short fragments and frequent strand exchanges [6] [5]. To further characterize the nature of the DNA synthesis occurring under these conditions, we used two-dimensional agarose-gel analysis. Using cells harboring the plasmid pBR322, we examined the structures and forms of the replicating molecules following irradiation in wild type and *uvrA* mutant cultures. Cultures were irradiated with a 50 J/m^2 , a dose which on average generates approximately 1 lesion per plasmid strand [12]. DNA was then purified from samples taken 0 and 15, 30, 45, 60 and 90 minutes after irradiation. The purified DNA was digested with PvuII, which cuts downstream of the plasmid's

unidirectional origin of replication to form a linear fragment, and the molecules were then analyzed using two-dimensional gel electrophoresis, which separates the DNA based on both size and structure. Non-replicating plasmids migrate as a linear 4.4 kb fragment, which appears as a prominent dot on the gel. Replicating plasmids form a Y-shape following PvuII digestion, migrate more slowly due to their larger size and non-linear shape and appear as an arc extending from the linear plasmid DNA. Following UV-exposure additional replication intermediates accumulate in a cone region extending from the arc and represent double Y and X-shaped structures with two branch points. In previous work, a subset of these molecules was shown to represent a transient regression of the replication fork that occurs prior to the resumption of replication. In wild type cells, these intermediates begin to resolve 30 minutes post-irradiation, coincident with the completion of lesion repair and recovery of replication [12]. In *uvrA* mutants, we observed that these intermediates accumulate to a higher degree than in wild type cultures and persist throughout the time course (Figure 2.2). In addition, higher order intermediates begin to accumulate above the cone region. The higher order intermediates range between 4, 8, and 16 times in size relative to the plasmid monomer and contain multiple crossovers, based on their retarded migration. In addition these molecules appear to contain regions of single stranded DNA, as evidenced by their resistance to restriction digestion. Thus, these observations are consistent with and confirm early studies suggesting that the post irradiation synthesis contains gapped DNA and involved elevated levels of strand exchanges [5, 6]. In addition, the two dimensional analysis demonstrates that this form of synthesis generates higher order intermediates containing multiple crossovers that persist and are unable to resolve into discrete plasmid molecules.

Assuming similar events occur in the chromosome, cells undergoing this form of recombination-dependent replication would produce chromosomes with duplications, deletions, and rearrangements that fail to faithfully resolve and segregate into daughter cells at the time of division. Thus, this form of aberrant replication may well account for the high rates of lethality observed in replicating *uvrA* mutants following low doses of UV-irradiation. Consistent with this view, a microarray analysis of the DNA content in UV-irradiated *uvrA* cells suggests unbalanced gene copy number throughout the chromosome. In contrast, the gene copy number remains balanced in post-irradiation synthesis occurring in wild type cells (see Appendix A).

Following low doses of UV-irradiation cells lacking nucleotide excision repair filament and contain DNA that fails to segregate into daughter cells.

In order to better understand the effect of the aberrant DNA synthesis observed in irradiated *uvrA* mutants on the fate of the cell, we examined the morphological changes in the cell and genome following UV-exposure by microscopy. Cells were treated with 6 J/m² and allowed to recover for four hours. The cells were then stained with 4,6-diamidino-2-phenylindole and observed using both brightfield and fluorescence microscopy. Following UV irradiation, wild type cells were seen to moderately filament (~ 2 cell lengths) during the first two hours after irradiation, but had returned to normal size within four hours. In UV-irradiated *recA* mutants, no filamentation occurred after either two or four hours of recovery. However, when we examined *uvrA* mutants following low doses of irradiation, we observed much more substantial filamentation. In many cases the filamentation was extensive (greater than three cell lengths) and cells

contained large, nucleoids that were unevenly distributed along the cell partitions. *uvrA6* mutants remained filamentous throughout the four-hour time course. The aberrant morphology observed in *uvrA6* mutants exposed to UV-irradiation indicates the replication occurring in these cells is not producing balanced chromosomes that are capable of segregating into daughter cells. The observations support the idea that lethality in the *uvrA* mutants likely results from aberrant unbalanced replication that occurs when the lesions are unable to be removed from the genomic template.

We have shown that the DNA synthesis following low doses of UV-irradiation in nucleotide excision repair deficient cells is coincident with a high rate of cell death. Furthermore, *uvrA* mutants exhibit a high rate of strand exchange and generate branched DNA molecules that do not resolve. These cells become grossly filamentous with large, irregularly distributed nucleoids following low doses of UV-irradiation, suggesting an inability to generate balanced chromosomes. Taken together these observations indicate that the DNA synthesis seen in nucleotide excision repair deficient cells is neither productive nor able to generate viable chromosomes. Rather than promoting survival, the DNA synthesis seen in these mutants seems to result from illegitimate recombination that leads to cell death. Consistent with this idea, preliminary microarray studies have shown that the DNA synthesis that takes place following low doses of UV irradiation is unbalanced and leads to a high degree of over replicated and under replication portions of the genome (Appendix A). These results suggest that even following these low doses of UV-irradiation, RecA is unable to promote survival in the absence of nucleotide excision repair. Instead, our results show that both RecA and UvrA are required for survival and the generation of viable chromosomes in this context. Cells that are proficient in

nucleotide excision repair but lack RecA are unable to survive low doses of UV light and do not exhibit any further DNA synthesis following irradiation. Following UV-exposure *recA* mutants undergo extensive DNA degradation initiating from replication forks [10, 11]. Furthermore, two dimensional agarose gel electrophoresis has shown that *recA* mutants do not accumulate intermediates associated with replication fork regression and maintenance¹². Consequently, the inability of *recA* mutants to survive low doses of UV-irradiation is likely due to an inability to protect and maintain replication forks that have been arrested by lesions. Together, these observations suggest that post replication recombination repair is insufficient to promote survival in the absence of nucleotide excision repair even following small amounts of UV-exposure and that, instead, RecA functions in the stabilization of replication forks arrested by UV-induced damage until these lesions are removed by nucleotide excision repair.

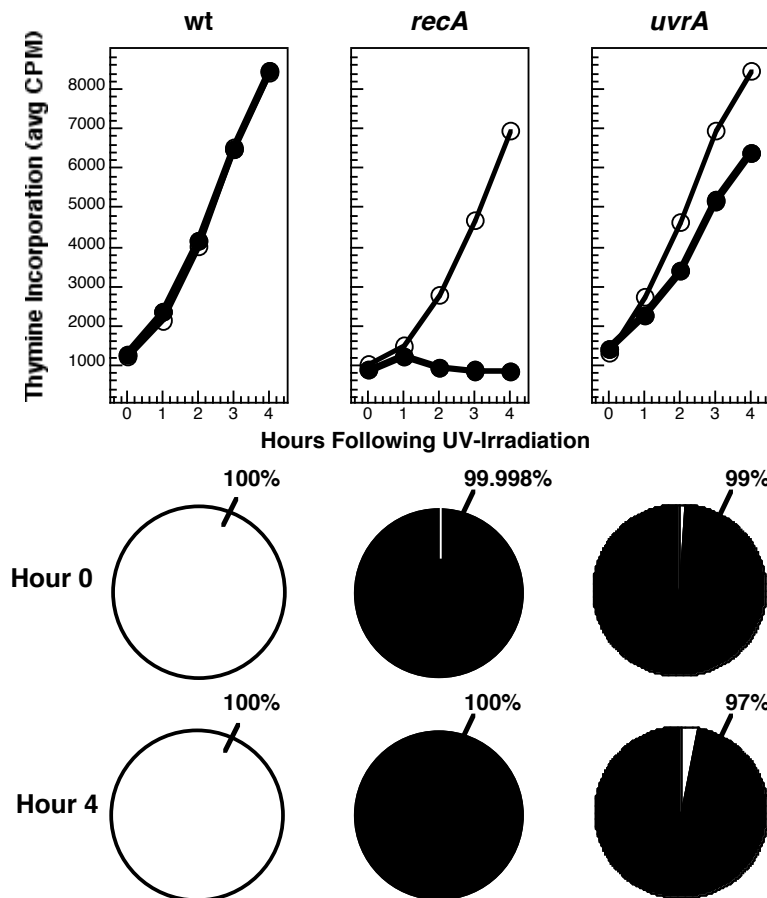


Figure 2.1 Cells deficient in nucleotide excision repair continue DNA synthesis following low dose UV-irradiation yet have significantly decreased viability. Cells were grown in the presence of [^3H] thymine and total DNA accumulation (average counts per minute between duplicate samples) is plotted. Cultures were irradiated with 6 J/m^2 (closed circles) or mock irradiated (open circles) at time zero. Pie charts represent the percent survival of samples taken concurrently with the incorporation data immediately following or 4 hours after UV-irradiation. The filled portion of each pie chart indicates the percentage of inviable cells.

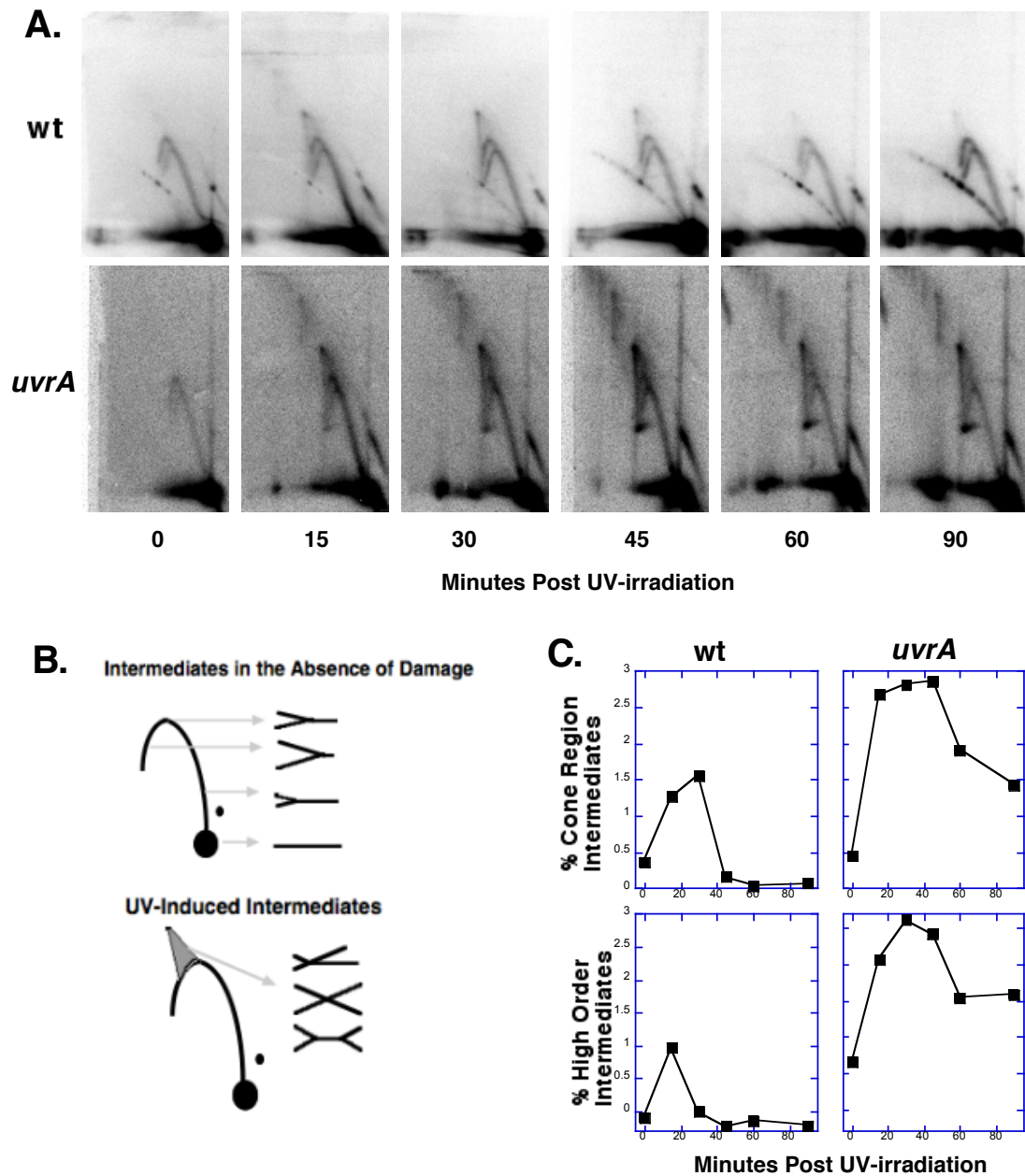


Figure 2.2 UV-induced structural intermediates accumulate and persist in cells unable to perform nucleotide excision repair. A. Cells containing the pBR322 plasmid were UV irradiated with 50 J/m². At the times indicated genomic DNA was purified, digested with PvuII, and the structural intermediates were examined by two-dimensional agarose gel analysis. Gels shown are representative of at least two independent experiments. B. Diagram of structural intermediates observed in the presence or absence of UV-induced damage. C. The percentage of UV-induced intermediates relative to non-replicating plasmids over time is plotted. Percentages were quantified as the ratio of radioactivity in either the cone region or the high-order intermediate region over the amount of radioactivity in the non-replicating region.

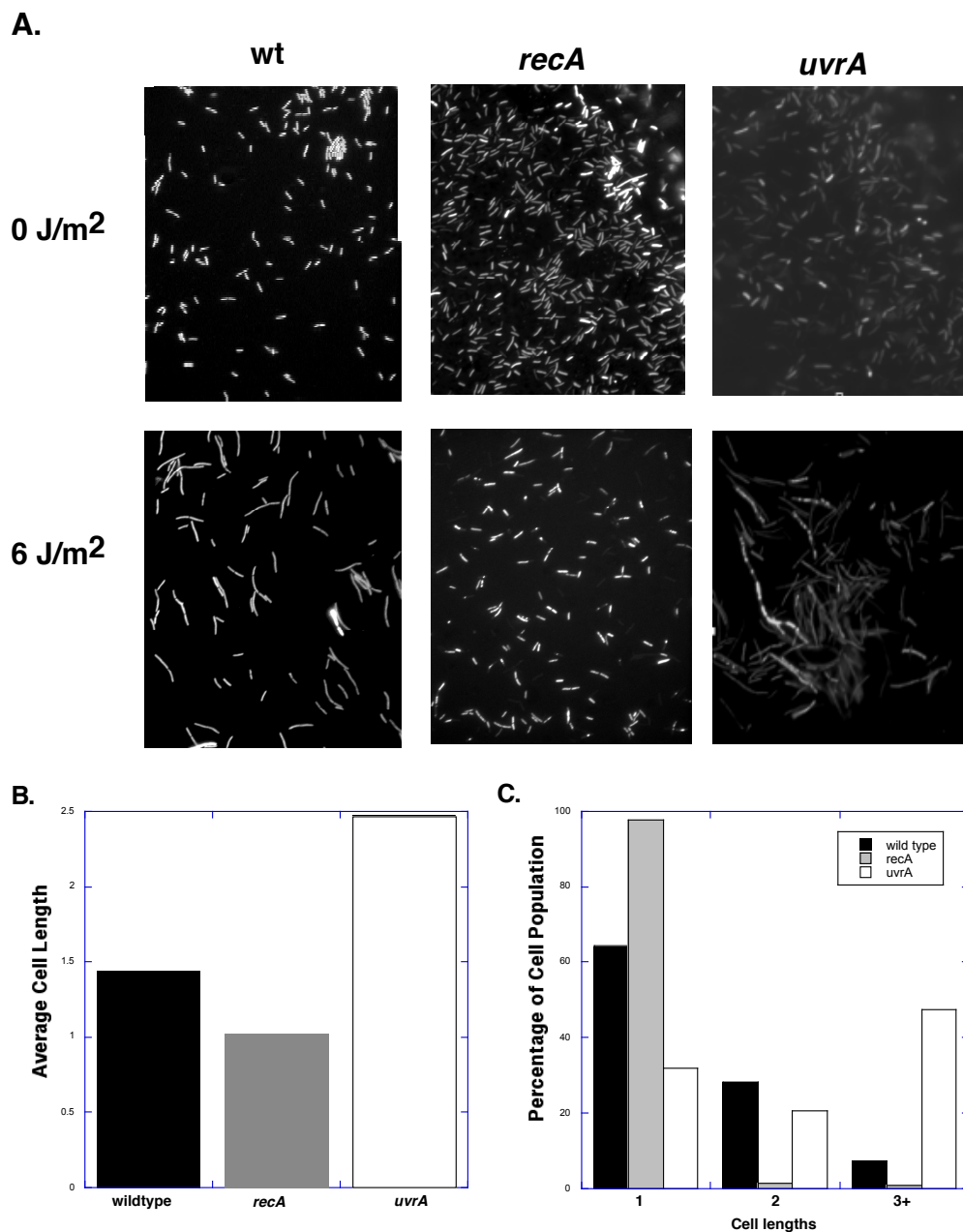


Figure 2.3 Following low doses of UV-irradiation, cells deficient in nucleotide excision repair exhibit morphologies distinct from both wild type cells and *recA* mutants. A. Cells were treated either mock-irradiated or treated with 6 J/m² at time 0. Following a 2-hour recovery period, cells were stained with DAPI and observed under a fluorescence microscope and photographed. B. Average cell length of each cell type two hours following irradiation. C. The percentage of cells of 1, 2, or greater than 3 cell lengths two hours after irradiation.

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CHAPTER III

UvrD PARTICIPATION IN NUCLEOTIDE EXCISION REPAIR IS REQUIRED FOR THE RECOVERY OF DNA SYNTHESIS FOLLOWING UV-INDUCED DAMAGE IN *ESCHERICHIA COLI*

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ABSTRACT

UvrD is a DNA helicase that participates in nucleotide excision repair and several replication-associated processes, including methyl-directed mismatch repair and recombination. UvrD is capable of displacing oligonucleotides from synthetic forked DNA structures *in vitro* and is essential for viability in the absence of Rep, a helicase associated with processing replication forks. These observations have led others to propose that UvrD may promote fork regression and facilitate resetting of the replication fork following arrest. However, the molecular activity of UvrD at replication forks *in vivo* has not been directly examined. In this study, we characterized the role UvrD has in processing and restoring replication forks following arrest by UV-induced DNA damage. We show that UvrD is required for DNA synthesis to recover. However, in the absence of UvrD, the displacement and partial degradation of the nascent DNA at the arrested fork occurs normally. In addition, damage-induced replication intermediates persist and accumulate in *uvrD* mutants in a manner that is similar to that observed in other nucleotide excision repair mutants. These data indicate that following arrest by DNA damage, UvrD is not required to catalyze fork regression *in vivo* and suggest that the

failure of *uvrD* mutants to restore DNA synthesis following UV-induced arrest relates to its role in nucleotide excision repair.

1. Introduction

The accurate duplication of the genome is critical to the survival of any organism. DNA damage, such as that caused by UV-irradiation, can disrupt the replication machinery and prevent it from completing its task [1, 2]. In *Escherichia coli*, a number of the cellular events associated with the recovery of replication forks arrested by UV-induced lesions are known to involve several gene products in the RecF pathway [3-5]. Following replication arrest, the nascent lagging strand of DNA is partially degraded through the coordinated activity of the RecJ nuclease and RecQ helicase [4]. The extent of degradation is limited by RecF-O-R, which facilitates loading and formation of a RecA filament at the stalled fork. Both biochemical and cellular studies suggest that RecF, -O and -R, together with RecA, facilitate strand exchange or regression at the branch point of the arrested fork [6,7]. Cellular studies suggest that this processing restores the lesion-containing region to a double-stranded form, allowing nucleotide excision repair to access and repair the lesion [6, 8]. In the absence of either processing or repair, the recovery is delayed and elevated levels of rearrangements, mutagenesis and lethality are observed [8- 10]. A number of other gene products have also been postulated to participate in aspects of the recovery process, but have yet to be examined *in vivo*.

UvrD is a DNA helicase that participates in both nucleotide excision repair and replication-associated processes. Nucleotide excision repair is the process by which bulky adducts and lesions are removed and repaired from DNA [11, 12]. During nucleotide excision repair (NER), a heterotetramer, UvrA₂UvrB₂, recognizes and binds the damaged

region [11, 13,14]. UvrD acts post-incision to release the resulting 10 to 12 bp oligonucleotide and UvrB-UvrC complex from the DNA [15-18]. The resultant gap is then filled in by DNA polymerase I and sealed by DNA ligase[19].

During replication, UvrD function is required to displace the nascent DNA strand during methyl-directed mismatch repair, a replication-coupled process that removes mispaired bases [20, 21]. It is required for replication of several rolling-circle plasmids [22], and co-purifies with DNA polymerase III holoenzyme under some conditions [23]. Conjugational and transformational recombination frequencies increase in *uvrD* mutants [24, 25] and decreases in strains overexpressing UvrD [26]. In addition, *uvrD* mutants are constitutively induced for the SOS response, and show elevated levels of RecA foci [27, 28].

The concept that UvrD may process replication forks following arrest comes from a number of genetic observations. *uvrD* mutants exhibit synthetic lethality with *rep* [29,30], which encodes another 3'-5' helicase that is required for the replication of phage Φ X174 and some plasmids [31, 32] and is postulated to remove obstacles on the DNA during replication such as bound proteins or transcriptional machinery [33, 34]. Viability in *uvrD rep* double mutants can be restored by mutations in *recF*, *recO* and *recR*, which are required to process and restore replication following arrest by DNA damage [5, 6, 35]. Subsequent studies found that purified UvrD was capable of displacing oligos and RecA filaments from synthetic replication fork structures *in vitro* [36, 37]. These observations led some researchers to speculate that, in addition to its other roles, UvrD function may participate in displacement of the lagging strand and RecA filament from arrested

replication forks [38,37]. However, the molecular function of UvrD at replication forks has not been directly examined *in vivo*.

Here we characterize the role of UvrD at the replication fork following arrest by UV-induced damage *in vivo*. We find that UvrD is necessary for DNA synthesis to resume following UV-irradiation. However, the initial degradation, processing, and regression of the arrested fork occur normally in the absence of UvrD. Similar to other mutants deficient in nucleotide excision repair, the regressed fork structures fail to resolve in *uvrD* mutants and continue to accumulate and persist. These observations indicate that UvrD is not required to catalyze fork regression *in vivo* and support the idea that the hypersensitivity and failure to restore replication in the absence of UvrD is likely due to its role in nucleotide excision repair.

2. Materials and Methods

Bacterial Strains

All bacterial strains used in this study are in an SR108 background, a *thyA36 deoC2* derivative of W3110 [39]. SR108, CL579 (SR108 *recF332::Tn3*), HL952 (SR108 *uvrA::Tn10*), HL1054 (HL108 *uvrD::tetR*), and HL944 (SR108 *recQ1803::Tn3*) have been described previously [4, 6, 8, 40]. CL1272 (DY320 *uvrD::kan*) was constructed using the recombineering strain DY329 [41]. The kanamycin resistance gene was amplified from Tn5 using PCR primers

5'CCCAACCTATTTTACGCGGCGGTGCCAATGGACGTTTCT-
ATGGACAGCAAGCGAACCG3' and

5'AGGCCAAATAAGGTGCGCAGCACCGCATC-

CGGCAACGTTATCAGAACTCGTCAAGAAG3'. The PCR product was then

transformed into DY329 to generate CL1272, selecting for kanamycin resistance. The gene replacement was transferred into SR108 using standard P1 transduction to generate CL1302 (SR108 *uvrD::kan*).

UV Survival Studies

Fresh overnight cultures were diluted 1:100 in DGCthy medium (Davis medium supplemented with 0.4% glucose, 0.2% casamino acids and 10 µg/ml thymine) and grown to an OD₆₀₀ of between 0.4 and 0.5 at 37°C in a shaking bath. Serial dilutions of each culture were plated in triplicate on Luria-Bertania plates supplemented with 10 µg/ml thymine and UV irradiated with the indicated doses. A Sylvania 15-watt germicidal lamp (254 nm) delivering an incident dose of 0.9 J/m²/s (0.2 J J/m²/s for doses less than 20 J/m²) was used for all irradiations. Plates were grown overnight at 37°C and colonies were counted the following day.

Recovery of DNA Synthesis

Fresh overnight cultures were diluted 1:100 in 50 ml DGCthy medium supplemented with 0.1 µCi/ml [¹⁴C]-thymine and grown to an OD₆₀₀ of 0.3 at 37°C in a shaking water bath. Half of each culture was mock irradiated and the other half was irradiated with an incident dose of 27 J/m². At the indicated times, duplicate 0.5-ml aliquots of each culture were pulse-labeled with 1 µCi/ml [³H]thymidine for 2 min at 37°. The cells were then lysed and the DNA precipitated using ice-cold 5% trichloroacetic acid (TCA). The DNA was filtered onto 2.4-cm Fisherbrand glass fiber filters and the amount of ¹⁴C and ³H was determined using a liquid scintillation counter.

DNA Degradation Assay

Fresh overnight cultures were diluted 1:100 in 6-ml DGCthy medium supplemented with 0.1 $\mu\text{Ci/ml}$ [^{14}C]thymine and grown to an OD_{600} of 0.3 in a shaking water bath at 37°C. At this point cultures were pulse labeled for 5 s with 1 $\mu\text{Ci/ml}$ [^3H]thymidine, filtered onto a 0.45-micron Millipore filter and rinsed twice with 1X NET buffer (100 mM NaCl, 10 mM EDTA, pH 8.0, 10 mM Tris, pH 8.0). The cells were then resuspended in 10 ml nonradioactive, pre-warmed DGCthy media, UV-irradiated at an incident dose of 27 J/m^2 and incubated in a 37°C shaking water bath. Triplicate 0.2-ml samples were taken at time zero, followed by duplicate samples every 20 min for the duration of the experiment. These samples were added to 5% TCA to lyse the cells and precipitate the DNA. The DNA was filtered onto 2.4-cm Fisherbrand glass fiber filters and the amount of radioactivity on each filter was measured using a liquid scintillation counter.

Two-dimensional Agarose Gel Electrophoresis

Cultures harboring the pBR322 plasmid were grown overnight in DGCthy medium in the presence of 100 $\mu\text{g/ml}$ ampicillin. One milliliter of this culture was pelleted, resuspended at a 1:100 ratio in 20 ml of DGCthy medium and grown without ampicillin to an OD_{600} of 0.5 at 37°C in a shaking water bath. The cultures were then UV-irradiated with 50 J/m^2 and 0.75-ml aliquots were transferred to an equal volume of ice-cold 2X NET (200 mM NaCl, 20 mM EDTA pH 8.0, 20 mM Tris, pH 8.0) at the times indicated. These samples were then pelleted, resuspended in 0.14 ml of lysis buffer (1.5 mg/ml lysozyme, 0.5 mg/ml RNase A in 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) and incubated at 37°C.

After 30 min, 10 µl of 20% Sarkosyl and 10 µl of 10 mg/ml proteinase K was added and the incubation was continued for 30 more min. The samples were then extracted twice with 4 volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and extracted once with 4 volumes of chloroform/isoamyl alcohol (24:1). The samples were dialyzed against 200 ml of TE buffer (2 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) for 1 h on floating 37-mm Whatman 0.05-µm pore discs and digested with PvuII overnight at 37°C. The samples were loaded onto 0.4% agarose gel following extraction with 2 volumes of chloroform/isoamyl alcohol (24:1) and run at 25 V for 16 hours in 1X TBE buffer (Tris-borate-EDTA, pH 8.0). For the second dimension, the lanes were excised, rotated 90 degrees, recast in a 1% agarose gel in 1X TBE and the gel was electrophoresed at 200 V for 7 h. The DNA from the gels was transferred to a HybondN+ nylon membrane by standard Southern blotting and the plasmid DNA was detected with an [α -³²P]dCTP (MP Biomedicals)-labeled pBR322 probe that was prepared using a nick translation protocol (Roche). Radioactivity was visualized and quantified using a Storm 840 Phosphoimager and ImageQuant software (GE LifeSciences).

3. Results

UvrD is required to restore DNA synthesis following arrest by UV damage.

We constructed a UvrD deletion, *uvrD::kan*, and compared its UV resistance along with a previously characterized strain, *uvrD::tet* (parental strain HL 1054 [40]) with that of *uvrA* and *recF* mutants (Figure 3.1). UvrA is required for the initial step of nucleotide excision repair and RecF is required for processing and maintaining forks arrested by UV-induced damage. Both *uvrD* mutations rendered cells more sensitive to

UV irradiation than wild-type cells. Consistent with previous studies, *uvrD* mutants are more sensitive than *recF* mutants [42], but less sensitive than *uvrA* mutants [43]. The higher resistance of *uvrD* compared with *uvrA* can be explained by its role in turnover of UvrC. *uvrD* mutants retain a limited ability to carry out nucleotide excision repair and remain proficient in repairing 6-4 photoproducts, which are removed preferentially before cyclobutane pyrimidine dimers or lesions in transcribed genes [40]. It is also important to note that *recF* mutants are able to withstand considerably more UV exposure than *uvrD* mutants. The RecF protein is involved in the stabilization of disrupted replication forks and, consequently, the susceptibility of *recF* mutants to UV damage is related to the frequency with which the replication machinery encounters a lesion. In the presence of nucleotide excision repair, the frequency of these events is substantially decreased. Therefore, that the *uvrD* mutant is considerably more sensitive than the *recF* mutant would indicate that the UvrD protein still plays a substantial role in nucleotide excision repair.

To determine if UvrD is required to resume DNA replication following arrest by UV-induced damage, we monitored DNA synthesis over time in UV-irradiated cultures of *uvrD* mutants. Cultures grown in the presence of ^{14}C -thymine were UV-irradiated or mock irradiated and allowed to recover over a period of 90 min. The rate of synthesis was monitored by pulse-labeling aliquots of the culture with ^3H -thymidine for two min at various times during the recovery period. In this manner, both the total DNA accumulation (^{14}C -incorporation) and the rate of synthesis (^3H -incorporation/2 min) can be followed simultaneously. By this assay in wild-type cells, the rate of synthesis dropped over 90% immediately following UV-irradiation and then began to recover after

approximately 20 min, and approached pre-irradiation levels by the end of the 90-min time course. A transient pause in the accumulation of DNA in the wild-type culture was also observed consistently at times prior to when replication resumed (Figure 3.2). For the purposes of comparison, we also examined mutants lacking RecF and UvrA, which have been shown previously to be defective in the resumption of replication following arrest by UV damage [5]. In these mutants, no further DNA accumulation was observed following irradiation and the rate of synthesis did not recover (Figure 3.2). When we examined UV-irradiated cultures of *uvrD*, we observed that the rate of DNA synthesis was inhibited to a similar extent as in *recF* and *uvrA* cultures after irradiation and also failed to recover (Figure 3.2). Additionally, no further accumulation of DNA was observed in these cultures. The results indicate that UvrD is necessary for the resumption of replication following arrest by UV-induced damage.

UvrD does not contribute to the nascent DNA processing that occurs following arrest at UV-induced damage.

Following the arrest of replication by UV-induced damage the nascent DNA at the replication fork is displaced and partially degraded prior to the resumption of replication[4]. Recent studies have postulated that UvrD may function in clearing and processing of blocked replication forks, which may account for its failure to restore DNA synthesis [38, 44, 45]. Alternatively, UvrD may function at forks blocked by UV damage specifically in a nucleotide excision repair capacity. To determine which roles of UvrD may be required in replication recovery following UV damage, we examined whether UvrD contributes to the displacement and degradation of the nascent DNA at replication

forks arrested by UV-induced damage. We reasoned that if the UvrD helicase were required to displace the nascent DNA, then the degradation of the nascent DNA at the arrested replication fork would be reduced in the protein's absence. To monitor DNA degradation, cultures grown in media containing ^{14}C -thymine were pulse-labeled for 5 s with ^3H -thymidine, collected on filters, resuspended in non-radioactive media, and immediately UV-irradiated. The amount of radioactivity remaining in the cultures was then followed over time. The dual radio-labeling allows us to simultaneously monitor the degradation that occurs in the total genomic DNA (^{14}C) and the nascent DNA synthesized immediately prior to irradiation (^3H). Following irradiation of wild-type cultures, the genomic DNA primarily remained intact and little or no degradation was detected (Figure 3.3). However, consistent with earlier studies, some limited degradation of the nascent DNA was detected at early times following irradiation [4, 8]. The loss of ^3H -labeled DNA ceased at a time that correlated with the resumption of DNA synthesis and then began to increase (Figure 3.3). In principle, an increase in ^3H should not be possible with this assay design. Previous work has shown that this increase is most likely due to remaining intracellular pools of radio-labeled thymidine that could not be washed away [5]. In the absence of RecF, which is required to limit the degradation at blocked replication forks, the nascent DNA degradation was more extensive and continued over a longer duration until approximately 50% of the nascent DNA has been degraded (Figure 3.3). In previous work, we have shown that the lagging strand is preferentially degraded following UV-irradiation. This may explain why the degradation ceases after half of the nascent DNA has been degraded [4]. For the purposes of comparison, we also examined the degradation occurring in *recQ* and *uvrA* mutants. RecQ is a helicase that has been

demonstrated to participate with RecJ to displace and degrade the nascent DNA at replication forks blocked by UV-induced damage [4]. In *recQ* mutants, no degradation of the nascent DNA was observed following irradiation and the remaining intracellular pools of ^3H -labelled thymidine were rapidly incorporated (Figure 3.3). UvrA is required for the initial recognition step of nucleotide excision repair and is not thought to play any role in processing of the replication fork. Following irradiation of *uvrA* mutants, we observed that the nascent DNA degradation still occurred, consistent with what has been reported previously (Figure 3.3 and [4, 8]). When we examined UV-irradiated cultures of *uvrD*, we observed that degradation of the nascent DNA occurred and was similar in extent to that seen in *uvrA* mutants (Figure 3.3). The data indicate that when replication is arrested by UV-induced damage, UvrD is not required for and does not contribute to the degradation of the nascent DNA *in vivo*.

UV-induced replication intermediates accumulate and persist in *uvrD* mutants.

To further differentiate between a potential role for UvrD in nucleotide excision repair and in processing replication forks, we compared the structural intermediates that are formed at replication forks following UV irradiation in *uvrD* mutants to *uvrA* and *recF* mutants. Previous work has shown that defects in nucleotide excision repair or replication fork regression, lead to different structural intermediates following arrest [6]. Intermediates were visualized on replicating molecules of the pBR322 plasmid using a two-dimensional (2D) gel electrophoresis technique. Replicating cells containing this plasmid were irradiated with 50 J/m^2 , a dose that produces approximately one lesion per plasmid strand [6]. Cells were harvested at various times after irradiation and the DNA

was purified and digested with PvuII, which linearizes the plasmid proximal to its unidirectional origin of replication. The replication intermediates were then examined using 2D agarose-gel electrophoresis and Southern analysis with ^{32}P -labeled pBR322 as a probe. In the absence of damage, non-replicating plasmids migrate as a linear 4.5-kb fragment, which forms the prominent large spot on the blot (Figure 3.4A). Replicating molecules, which form Y-shapes, migrate more slowly due to their increased size and non-linear shape and appear as an arc extending out from the spot of linear plasmid fragments. Following irradiation of wild-type cultures, a transient cone region is observed above the arc of replicating Y-shaped molecules, consisting of X-shaped and double-Y shaped molecules (Figure 3.4). In previous work, we demonstrated that a portion of these molecules represent products that were formed by a RecF-catalyzed regression of the replication fork DNA [3, 6, 46]. These damage-induced intermediates begin to resolve after 30 min, at a time that correlates with the removal of lesions and the recovery of replication. In the absence of RecF, the arrested fork DNA is not maintained and these intermediate structures are not observed (Figure 3.4). By contrast, in *uvrA* mutants, the fork regression occurs normally but fails to resolve as the obstructing lesion is not removed from the DNA. In these mutants, the regressed fork intermediate is seen to persist and accumulate, forming higher-order, illegitimate intermediates by the end of the 90-min time course (Figures 4B and 4C).

We reasoned that if UvrD was required to catalyze the regression of the fork DNA at UV-induced lesions, then the cone region intermediates would be reduced or absent in these mutants following UV irradiation. However, when we examined *uvrD* mutants, we observed elevated levels of these intermediates that accumulated throughout the 90-min

time course (Figure 3.4). These intermediates went on to form the higher-order intermediates that are a hallmark of nucleotide excision repair-deficient mutants, consistent with the high levels of recombination and strand exchange seen in these mutants [9, 10]. The presence of the fork regression products in *uvrD* mutants indicates that UvrD is not required to catalyze this reaction *in vivo*. Further, the similarity between the intermediates seen in *uvrA* and *uvrD* mutants would suggest that the failure of *uvrD* mutants to resume DNA synthesis after UV irradiation is most likely due to their inability to carry out nucleotide excision repair.

4. Discussion

In addition to its role in nucleotide excision repair, UvrD has also been postulated to catalyze fork regression and the displacement of the nascent lagging strand during the recovery of replication after arrest [44, 45, 36]. Here, we examined the functional roles for UvrD's contribution to cell survival and the recovery of replication following arrest by UV-induced damage. We observed that both the nascent strand processing and regression of the fork DNA occurs normally in the absence of UvrD. Rather than diminished levels of regressed fork intermediates forming in *uvrD* mutants, we observed that elevated levels of these intermediates formed and accumulated, similar to that seen in other nucleotide excision repair mutants. The observations are most consistent with the idea that the failure to restore replication in UvrD mutants is due to its role in nucleotide excision repair.

A role of UvrD in nucleotide excision repair, by itself, could sufficiently account for the hypersensitive and replication-defective phenotypes observed in *uvrD* mutants after UV irradiation. UvrD is required for the turnover of UvrC, which is not upregulated

during the SOS response [47]. Thus, only a limited amount of repair occurs in the absence of UvrD, which is generally restricted to the repair of 6,4-photoproducts [40]. The minimal amount of nucleotide excision repair seen in *uvrD* mutants is consistent with it being modestly more resistant to UV damage than other repair mutants of this class. Otherwise, with respect to the processing of the nascent DNA, fork reversal, and impaired recovery of replication, *uvrD* mutants exhibit phenotypes nearly identical to those of other nucleotide excision repair mutants.

The concept that UvrD may function in displacing the nascent DNA and promote fork reversal following arrest developed from a number of indirect genetic observations. A series of previous studies observed that in *recBC* mutants, which are defective in double-strand break repair, elevated levels of chromosome breaks can be detected in thermo-sensitive replication mutants, *dnaE* and *dnaN* (the catalytic subunit of Pol III and the Pol III clamp, respectively [48, 49]) at the restrictive temperature [37, 44, 45]. If cells were additionally mutated in *uvrD*, the level of detectable chromosome breaks was reduced. The authors speculated that these chromosome breaks arose as a result of replication forks collapsing to generate double-strand breaks. However, the assays employed in these studies were unable to address where the breaks form in the chromosome, and other studies have suggested that breaks repaired by RecBC do not form directly at the replication forks following arrest *in vivo* [4, 5,50]. Curiously, these studies also noted that a different *uvrD* mutant lacking both ATPase and helicase activity failed to suppress chromosome breaks in these backgrounds.

When considering the differences between the results obtained in these studies, it is also important to consider the mechanism by which replication is arrested in each case.

Whereas we used UV-induced damage to block the replication machinery, studies observing chromosomal breaks have often disrupted the replisome proteins themselves, using thermosensitive mutants. It seems probable that the biological events occurring after the loss of replication proteins would be distinct from those that occur when replication is blocked by impediments such as proteins or lesions, especially if one assumes that the replication proteins are required for the natural recovery process. Consistent with this, previous work from our lab has demonstrated a marked difference between the events following replication arrest caused by UV-induced damage and disruption of the DnaB helicase [51]. Whereas replication forks blocked by UV-induced lesions are protected and maintained by the RecFOR proteins, disruption or loss of DnaB helicase results in the collapse and degradation of the replication fork, a process that is antagonized by RecFOR function [51].

Other genetic studies have inferred a role for UvrD in processing replication forks based on the synthetic lethality between *rep* and *uvrD* mutants [29, 35]. The Rep helicase is suggested to play a role in removing nucleoproteins, DNA secondary structures, or transcriptional machinery encountered by the replisome during replication[34, 52]. These observations have been interpreted to suggest that UvrD may be partially redundant with Rep function in removing nucleoprotein impediments encountered during replication [34]. However, both Rep and UvrD are both directly associated with replication processes and it is unclear whether the synthetic lethality of *rep uvrD* double mutants can be attributed to the inability to overcome transcriptional blocks to replication or as a result of other impediments.

We have shown that when replication is blocked by UV-induced damage, it does not contribute to the displacement of the lagging strand or replication fork reversal, but is required to carry out nucleotide excision repair before replication can resume. We do not rule out the possibility that UvrD contributes to fork processing when replication encounters other impediments, such as DNA-bound proteins, RNA polymerases, or even other forms of damage. It would be of interest to pursue these investigations in future studies as well as address how UvrD can generate chromosome breaks in the unusual case where replication proteins are targeted for disruption using thermosensitive mutants.

Acknowledgements

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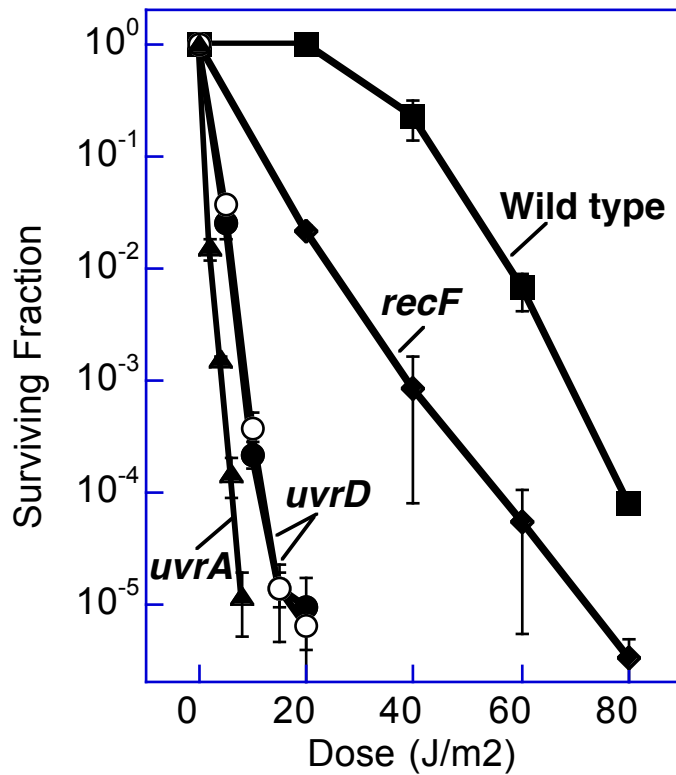


Figure 3.1 Cells lacking UvrD are hypersensitive to irradiation with UV light. Survival of wild-type (■), *recF* (◆), *uvrA* (▲), *uvrD::kan* (●), and *uvrD::tet* (○) cultures following irradiation with the indicated UV doses. Error bars represent the standard error of the mean.

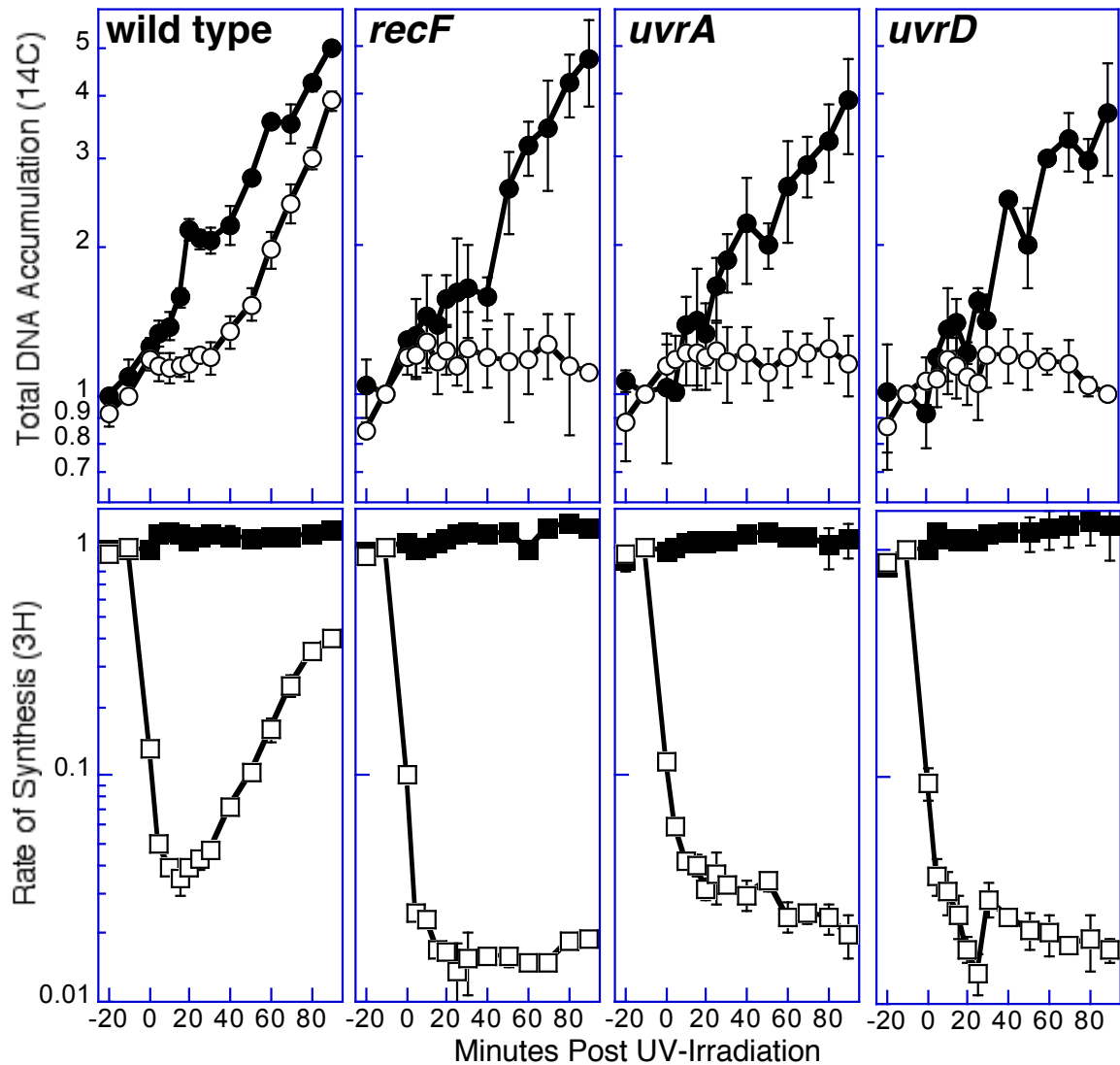


Figure 3.2 UvrD is required for the recovery of replication following UV irradiation, but not for replication in the absence of damage. Cells grown in the presence of [¹⁴C]-thymine were pulse-labeled for 2 min with [³H]-thymidine at the times indicated following either UV irradiation with 27 J/m² (open symbols) or mock irradiation (closed symbols). Total DNA accumulation (¹⁴C incorporation, circles) and rate of synthesis (³H incorporation/2 min, squares) are plotted. Graphs represent the average of at least three independent experiments. Error bars represent the standard error of the mean. The level of [³H] and [¹⁴C] in preirradiated DNA ranged between 30,000-50,000 cpm and 3000-6000 cpm for all experiments.

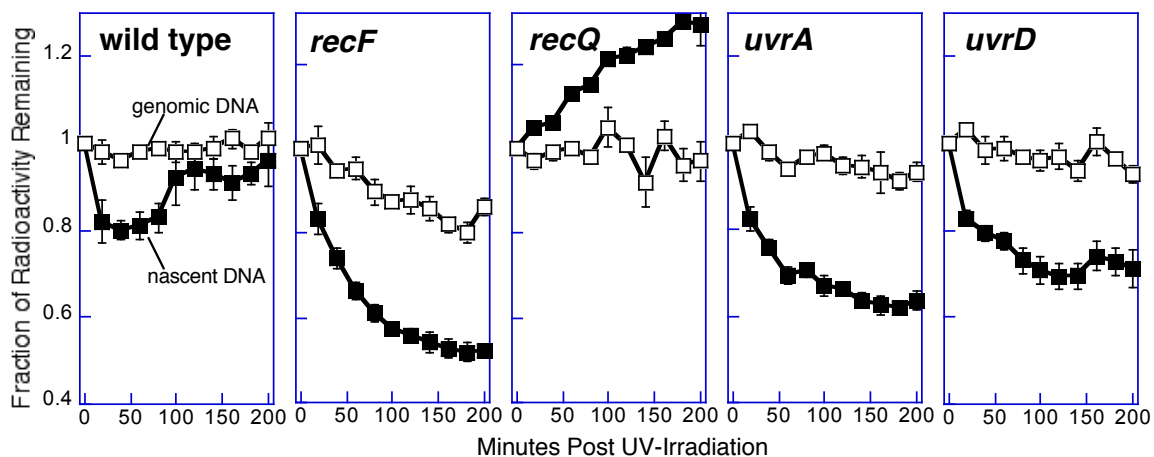


Figure 3.3 In the absence of UvrD, the nascent DNA at stalled replication forks is degraded in a manner similar to other repair mutants. [^{14}C]-thymine labeled cultures were pulse labeled with [^3H]-thymidine for 5 s before the cells were collected, resuspended in non-radioactive media and UV irradiated with 27 J/m^2 . The fraction of ^{14}C -labeled genomic DNA (□) and ^3H -labeled nascent DNA (■) remaining over time is plotted. Graphs represent the average of three independent experiments. The level of [^3H] and [^{14}C] in DNA immediately preceding irradiation ranged between 2500-7000 cpm and 1000-2500 cpm in all experiments. Error bars represent the standard error of the mean.

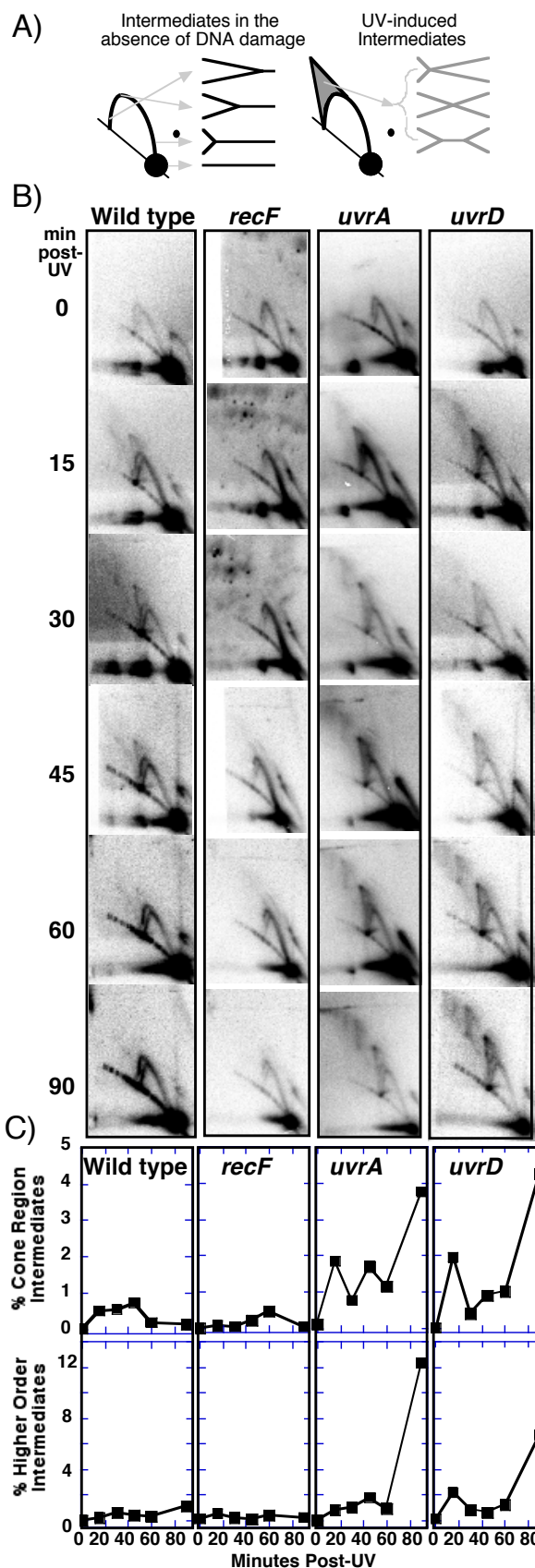


Figure 3.4 In the absence of UvrD, blocked replication forks persist leading to the accumulation of higher-order recombination intermediates in a manner similar to *uvrA* mutants. A) Diagram of structural intermediates observed in the presence or absence of UV-induced damage. B) Cells containing the pBR322 plasmid were UV-irradiated with 50 J/m². At the times indicated, genomic DNA was purified, digested with PvuII, and the structural intermediates were examined by two-dimensional agarose gel analysis. Gels shown are representative of at least two independent experiments. C) The percentage of UV-induced intermediates relative to non-replicating plasmids over time is plotted. Percentages were quantified as the ratio of radioactivity in either the cone region or the high-order intermediate region over the amount of radioactivity in the non-replicating region.

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APPENDIX UNBALANCED DNA SYNTHESIS FOLLOWING UV-IRRADIATION IN A NUCLEOTIDE EXCISION REPAIR MUTANT

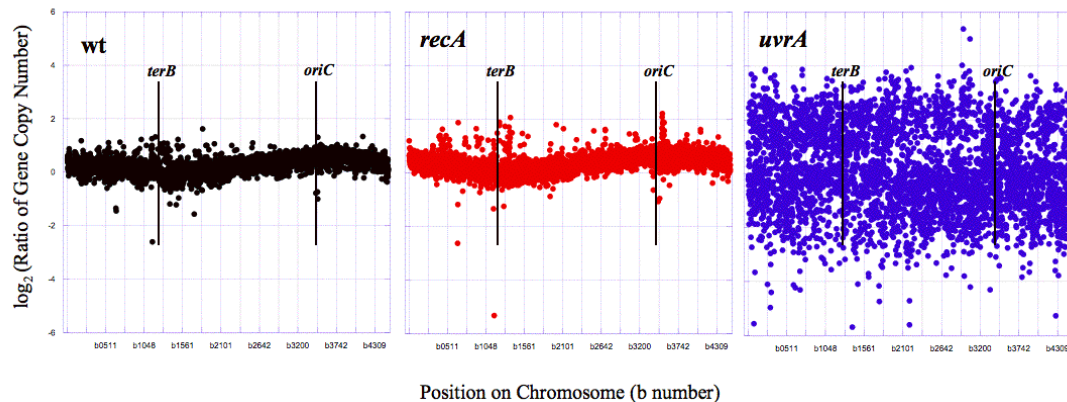


Figure A.1 DNA synthesis following low doses of irradiation in *uvrA* mutants is unbalanced and results in over- and under replicated regions of the chromosome. Microarray analysis was utilized to compare the copy number of each gene around the chromosome in cells that have recovered for 4 hours following UV-irradiation to that of the cells prior to UV-irradiation. Specifically, the log base 2 of the ratio of gene copy number prior to irradiation to the gene copy number 4 hr following irradiation is plotted against the gene's position on the chromosome. The positions of the termination signal, *terB*, and origin of replication, *oriC*, are shown for reference. In wild type cells, the genes are processively copied around the chromosome as replication remains balanced following irradiation. Following UV-irradiation in *recA* mutants, no further DNA synthesis is observed and, therefore, the copy number of each gene remains the same before and after UV-irradiation. *uvrA* mutants continue to synthesize DNA following low doses of irradiation. However, this synthesis is not evenly distributed and results in an uneven copy number as some regions of the chromosome are over replicated and others are under replicated.